STIC-ILL

From: Scheiner, Laurie
Sent: Thursday, September 13, 2001 3:49 PM
To: STIC-ILL
Subject: ref request (816,531)

Morimoto et al., Vaccine, (14 May, 2001) vol. 19, No. 25-26, pp. 3543-3551. Thanks!

Laurie Scheiner
Art Unit 1648
CM1 8E05
308-1122
8E12

ELSEVIER

Vaccine 19 (2001) 3543–3551

Vaccine

www.elsevier.com/locate/vaccine

Genetic engineering of live rabies vaccines

Kinjiro Morimoto [a], James P. McGettigan [a,b], Heather D. Foley [a,b], D. Craig Hooper [a], Bernhard Dietzschold [a], Matthias J. Schnell [b,c,*]

[a] *Departments of Microbiology and Immunology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA*
[b] *The Dorrance H. Hamilton Laboratories, Center for Human Virology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA*
[c] *Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA*

Received 26 September 2000; received in revised form 12 December 2000; accepted 2 January 2001

Abstract

Rabies virus is not a single entity but consists of a wide array of variants that are each associated with different host species. These viruses differ greatly in the antigenic makeup of their G proteins, the primary determinant of pathogenicity and major inducer of protective immunity. Due to this diversity, existing rabies vaccines have largely been targeted to individual animal species. In this report, a novel approach to the development of rabies vaccines using genetically modified, reverse-engineered live attenuated rabies viruses is described. This approach entails the engineering of vaccine rabies virus containing G proteins from virulent strains and modification of the G protein to further reduce pathogenicity. Strategies employed included exchange of the arginine at position 333 for glutamine and modification of the cytoplasmic domain. The recombinant viruses obtained were non-neuroinvasive when administered via a peripheral route. The ability to confer protective immunity depended largely upon conservation of the G protein antigenic structure between the vaccine and challenge virus, as well as on the route of immunization.

1. Introduction

Rabies is a worldwide public health problem. While in most developing countries dogs represent the major rabies reservoir, in North America, reservoirs of rabies exist in many diverse animal species [1,2]. The most frequently reported rabid wildlife species in the USA are raccoons (50.5%) followed by skunks (24.0%) [2]. Outbreaks of rabies infections in these terrestrial mammals are found in broad geographic areas in the United States [1]. Oral immunization of stray dogs and wildlife against rabies is the most effective method to control and eventually eradicate rabies [3]. In this regard, significant progress has been made in the development of oral rabies vaccines for the control of vulpine rabies [4]. However, while oral immunization with conventional modified-live vaccines such as SAD B19, SAG-2, or poxvirus-rabies glycoprotein recombinant vaccines are very effective in foxes [4], they do not immunize skunks or induce only low seroconversion by the oral route [5]. Moreover, very high doses of these vaccines are necessary to induce protective immunity after oral immunization of dogs [6] which makes an oral field vaccination, for economical reason, impractical. Therefore, in order to control wildlife rabies, more potent, cost-effective live rabies vaccines must be developed.

In addition to virus-neutralizing antibody (VNA), which are believed to be the major immune effectors against rabies [7], rabies virus antigen-specific $CD4^+$ and $CD8^+$ T cells [8], as well as innate mechanisms [9] play an important role in the immune defense against rabies. Since VNA is induced by the rabies virus G and cellular responses such as $CD8^+$ T cells are predominantly triggered by the internal rabies virus proteins, live rabies virus represents the best immunogen that can confer optimal protective immunity after a single inoculation. However, a live rabies vaccine that can be used under field conditions must be safe for humans, the target species, and any other animal species that can come in contact with the vaccine. Therefore, to be useful as a vaccine strain, pathogenicity of the virus

* Corresponding author.
E-mail address: matthias.schnell@mail.tju.edu (M.J. Schnell).

0264-410X/01/$ - see front matter
PII: S0264-410X(01)00064-0

must be highly attenuated without affecting the antigenic and immunogenic properties of the virus.

It was earlier shown that the rabies virus G is a major contributor to the pathogenicity of the virus [10–12]. Several G-associated pathogenic mechanisms have been identified, for e.g. (1) G must interact effectively with cell surface molecules that can mediate rapid virus uptake [13–16]; (2) G must interact optimally with the RNP-M complex for efficient virus budding [17–19]; and (3) expression levels of G must be controlled to prevent over-expression, which causes functional impairment of the infected neuron [11]. We used different modified rabies virus G genes to engineer rabies recombinant viruses, which exhibited a marked decrease in virus uptake or virus egress and a higher replication efficiency and higher G expression levels than wild-type viruses [19]. These recombinant viruses were found to be nonpathogenic after intramuscular inoculation but differed greatly in their ability to induce protective immunity.

2. Materials and methods

2.1. Viruses

CVS-N2c and CVS-B2c are subclones of the mouse-adapted CVS-24 rabies virus [20]. SHBRV-18 virus is a silver-haired bat-associated street rabies virus strain and DRV-7 is a dog-associated street rabies virus strain both isolated from human rabies victims [21]. SN-10 is a non-pathogenic virus strain derived from SAD B19 [19,22]. The generation of the recombinant rabies viruses R-B2c, R-N2c, and R-SHB18 is described elsewhere [19]. To obtain SN10-333, R-N2c-333, and R-B2c-333, arg 333 of G was replaced with gln by site-directed mutagenesis. To construct R-N2cT recombinant virus cDNA clone containing replaced sequences of the G cytoplasmic domain, a cDNA fragment corresponding to the ectoplasmic domain of CVS-N2c G was amplified with the CVS-Sma5 primer (5′-TTTCCCGGGAAGATGGTTCCTCAGGTTCTTTTG-3′; SmaI site in boldface, start codon underlined) and N2C-Afl3 primer (5′-CTTCTGCAACATGTCATTAGGGAAAATATC-3′; AflIII site in boldface; this primer introduced an AflIII restriction site in cytoplasmic domain of the CVS-N2c G gene), and was ligated with the cDNA fragment corresponding to the cytoplasmic domain (AflIII site) of SHBRV-18 G gene. The ligated fragment was amplified with primers CVS-Sma5 and B18-Nhe3 (5′-GGGCTAGCTCACATCCCGGTCTCACTTT-3′; NheI site in boldface, stop codon underlined). The PCR product was cloned into the SmaI and NheI sites of pSN plasmid and the resulting plasmid was designated pR-N2cT. Recombinant viruses were rescued as described [19].

2.2. Virus infectivity assay

Infectivity assays were performed at 34 or 37°C on monolayers of NA cells in 96-well plates as earlier described [23]. All titrations were carried out in triplicate.

2.3. Determination of neutralizing antibody (VNA)

The CVS-B2c, CVS-N2c, and SHBRV-18 virus stocks were calibrated to 10^5 focus forming units (FFU) per ml and then treated with serial dilutions of mouse sera. The neutralization test was performed as described [23]. Neutralization titers were defined as the inverse of the highest serum dilution that neutralizes 50% of the challenge virus.

2.4. Analysis of rabies virus RNP-specific antibodies in ELISA

RNP-specific antibodies were assessed in solid ELISA. Plates (PolySorb™, Nunc) were coated at room temperature in a humidified chamber overnight with 5 μg/ml rabies virus nucleoprotein diluted in phosphate-buffered saline (PBS). The plates were blocked with 5% powdered milk in PBS containing 0.05% Tween20 (PBS–Tween) prior to the addition of serum samples. Following incubation at room temperature for 2 h, the plates were washed three times to remove unbound primary antibody followed by the addition of horseradish peroxidase-conjugated (HRP) goat anti-mouse-IgG (H + L) secondary antibody (1:5000, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After 1 h incubation at room temperature, plates were washed three times and 200 μl OPD-substrate (*o*-phenylenediamine dihydrochloride, Sigma) was added to each well. The reaction was stopped by the addition of 50 μl of 3 M H_2SO_4 per well.

Absorbance values were read in a microplate spectrophotometer (Biotek, Winooski, VT) at 450 nm.

2.5. Pathogenicity studies in mice

Groups of ten 8–10 week-old female Swiss Webster mice (Taconic Farms, NY) were infected intracranially (i.c.) with 10 μl containing 10^4 infectious particles or into the gastrocnemius muscle (i.m.) with 100 μl containing 10^6 infectious particles of the different virus strains. After infection, the mice were observed daily for 4 weeks and the mortality was calculated from the percentage of surviving animals in each group. Rag-2 mice: Groups of ten mice were injected with 100 μl containing 10^6 FFU of SN10, SN10-333, R-B2c-333 into the gastrocnemius muscle. Mice injected with PBS served as controls.

2.6. Immunization and virus challenge

Groups of ten 8–10 week-old female Swiss Webster mice (Taconic Farms, NY) were inoculated i.m. with 100 μl of serial 1:10 dilutions of live rabies recombinant viruses and 10 days later the animals were infected i.c. with 10 μl containing 100 i.c. LD_{50} of CVS-N2c, DRV-7, or SHBRV-18 challenge virus. The mice were then observed for 4 weeks for the development of clinical signs. The 50% effective dose (ED_{50}) was calculated as described [24]. To immunize mice orally, 50 μl containing 10^6 FFU of recombinant virus was instilled into the buccal cavity of groups of ten mice and mice were eye-bled 2 weeks later and challenged with 100 LD_{50} of CVS-N2c i.c. Mice were euthanized at onset of neurological signs (paralysis).

2.7. Modified NIH test for potency

Groups of ten mice were immunized with different dilution of the vaccine under test. Ten days after vaccination, the immunized animals and a control group of mice are challenged i.c. with the respective challenge virus as indicated.

2.8. Statistics

VNA and ELISA titers are indicated as arithmetic mean titer and standard errors are indicated as error bars. Titers within the groups were compared using two-sample t-test (Student's t-test) and statistical significant differences ($P < 0.05$) are indicated by a star.

3. Results

3.1. Pathogenicity of rabies recombinant viruses

We recently reported the generation and phenotypic characterization of several recombinant rabies viruses that could potentially be useful for vaccination [19]. The rabies virus glycoproteins of three highly neuroinvasive and neurotropic RV strains SHBRV-18, CVS-N2c and CVS-B2c were introduced into the RV vaccine strain SN-10. The new viruses were designated R-SHB18, R-N2c and R-B2c (Fig. 1). Even though pathogenicity of all three recombinant viruses was markedly lower than that of the wild-type viruses, R-N2c and R-B2c were still pathogenic after peripheral administration in mice (Fig. 2A). It has been earlier shown that an exchange of an arginine at position 333 to glutamine within the RV G protein attenuates certain RV strains [4,10]. We, therefore, introduced this mutation within the G protein of R-B2c and R-N2c (R-N2c-333 and R-B2c-333, see Fig. 1). In contrast to R-B2c-333, the same mutation in RN2c-333 G did not result in a reduction of pathogenicity (Fig. 2A). We, therefore, applied another method to attenuate R-N2c. Our earlier results indicated that the attenuation of R-SHB18 resulted from a delay in virus production, probably caused by a sub-optimal interaction of the SHB18 G cytoplasmic tail with the internal viral proteins of SN-10. We, therefore, exchanged the cytoplasmic domain of the R-N2c glycoprotein with that of the SHB18 G protein resulting in the recombinant RV R-N2cT. As expected, a reduced budding efficiency was observed for

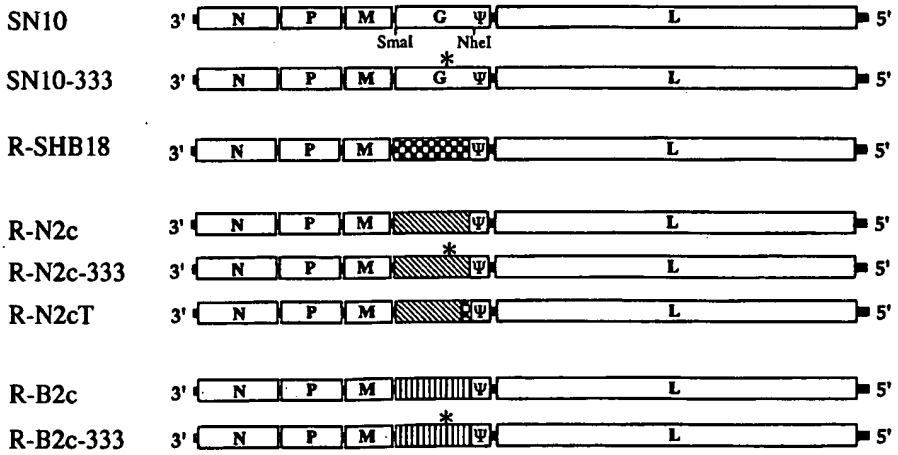

Fig. 1. Diagram showing the design of recombinant rabies virus. Using reverse genetics, the G protein gene of the SN-10 strain (SN10) was removed and replaced by the G protein gene of the SHBRV-18, CVS-N2c, or CVS-B2c, resulting in the recombinant viruses R-N2c, R-B2c, and R-SHB18. R-N2cT was obtained by replacing the cytoplasmic tail of its G protein with that of the SHBRV-18 G protein. * indicates the introduction of an arg→gln mutation at position 333 of the G protein of SN10 (SN10-333, R-N2c (R-N2c-333), and R-B2c (R-B2c-333).

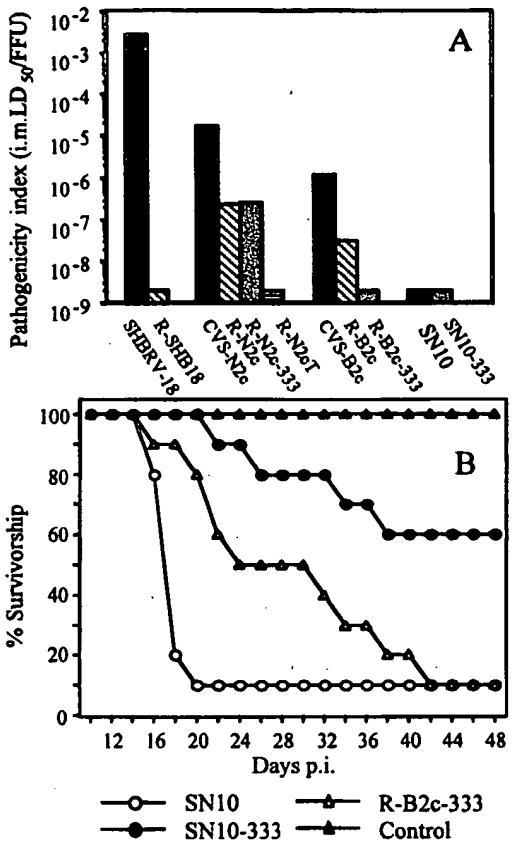

Fig. 2. (A) Pathogenicity indices of rabies recombinant viruses and the corresponding parental viruses. Pathogenicity index was defined as intramuscular LD_{50} determined in 8-week-old Swiss Webster mice divided by the virus titer determined in mouse neuroblastoma (NA) cells. (B) Pathogenicity of rabies recombinant viruses in Rag-2 mice. Groups of ten mice were injected with 100 μl containing 10^6 FFU of SN10, SN10-333, R-B2c-333 into the gastrocnemius muscle. Mice injected with PBS served as controls.

R-N2cT compared with R-N2c (data not shown) resulting in a substantial reduction of the pathogenicity index (Fig. 2A). If a direct i.c. administration is used, even attenuated viruses, which do not kill when injected via a peripheral route, cause lethal disease in the majority of the infected animals [10,12]. As can be seen in Table 1, this is the case for R-SHB18, R-N2cT, and SN10. In contrast, i.c. administration of 10^3 infectious particles of R-B2c-333 caused only 10% mortality and an SN10 variant with an $arg^{333} \rightarrow gln^{333}$ mutation within the G protein (SN10-333) was completely non-pathogenic. The strong reduction in the pathogenicity of the arg333 mutants is further seen after i.m. infection of Rag-2 mice, which are deficient in both T and B cells (Fig. 2B). While 90% of Rag-2 mice injected with the RV vaccine strain SN10 succumbed to infection within 18 days p.i., 80% of the SN 10-333 infected mice and 50% of the R-B2c-333 infected mice were still healthy and showed no loss of body weight 30 days p.i. We were unable to isolate virus or to detect viral RNA in Rag-2 mice, which survived the infection (data not shown).

Table 1
Pathogenicity of rabies recombinant viruses

Vaccine strain	% Mortality	
	i.m.[a]	i.c.[b]
R-SHB18	0	90
R-N2cT	0	90
R-B2c-333	0	10
SN10	0	100
SN10-333	0	0

[a] Groups of 20 mice were injected with 10^3 FFU into the gastroenemius muscle.

[b] Groups of 20 mice were injected with 10^3 FFU into the brain.

3.2. Antibody responses following intramuscular vaccination with attenuated rabies viruses

Next, we analyzed the four most attenuated recombinant RVs R-SHB18, R-N2cT, R-B2c-333 and SN10-333 for their usefulness as a live-attenuated RV vaccine. Since all of these viruses share the same proteins except for G, we assessed the antibody response to the RNP as a general index of virus replication with respect to antigenic mass and immunogenicity (RNP, Fig. 3A). The amount of α-RNP antibodies most likely parallels the quantity of viral antigens produced after immunization [25]. Anti-RNP titers obtained with the different vaccine strains were similar in mice immunized i.m. with the higher vaccine concentrations (5×10^6 and 5×10^5 FFU). These data suggest that the replication efficiency of the vaccine strains used showed no statistical significant difference after i.m. immunization. However, in the case of oral immunization, the induction of anti-RNP antibody titers depended largely on the virus used for vaccination. Lowest anti-RNP antibody titers were seen in the group of mice immunized with R-SHB18, indicating that this virus replicates poorly after oral application (Fig. 4A).

Since rabies virus VNA are considered to be the major immune effectors against rabies [7,8], we next determined VNA titers in mice immunized with different doses of our recombinant RVs (R-SHB18, SN10-333, R-B2c-333, and R-N2cT) against tissue culture-adapted rabies virus strains that are known to have distinct G structure [CVS-B2c (Fig. 3B), CVS-N2c (Fig. 3C), and SHBRV-18 (Fig. 3D)]. The results show that at the higher vaccine concentrations (5×10^6 and 5×10^5 FFU), all four recombinant viruses induced VNA titers against all three in vitro challenge viruses. Differences in VNA titers were observed at the lower doses of recombinant viruses (5×10^3 and 5×10^2 FFU). In this case, VNA titers of sera from mice immunized with R-N2cT were highest against the homologous CVS-N2c (Fig. 3C). On the other hand, the

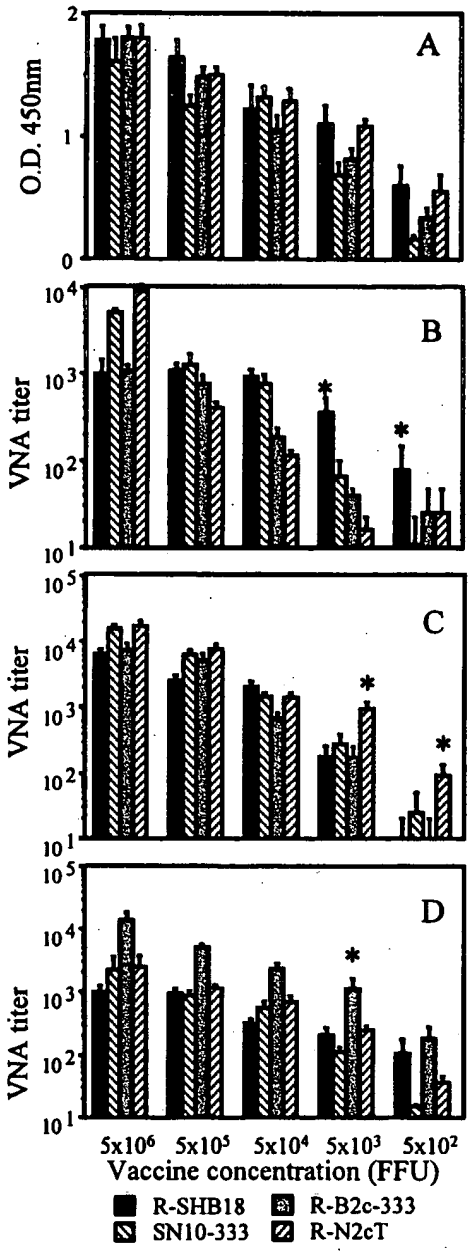

Fig. 3. Anti-RNP ELISA and VNA titers in mice immunized with live rabies recombinant viruses. (A) Groups of ten 8-week-old Swiss Webster mice were inoculated i.m. with different concentrations of R-SHB18, SN10-333, R-B2c-333 and R-N2cT. Mice were bled 10 days after immunization and ELISA titers were determined in 96-well plates coated with SN10 RNP. (B-D) VNA titers were determined on NA cells grown in 96-well plates, using CVS-B2c (B), CVS-N2c (C), or SHBRV-18 (D) as challenge viruses. VNA and ELISA titers are indicated as arithmetic mean titer from ten mice and standard errors are indicated as error bars. Titers within the groups were compared using two-sample t-test and statistical significant differences ($P < 0.05$) are indicated by a star.

highest VNA titers against SHBRV-18 were produced in mice immunized with R-B2c-333 (Fig. 3D). While oral immunization with SN10-333, R-B2c-333, and R-N2cT also resulted in the production of high VNA titers, the same amount of R-SHB18 vaccine induced only very low VNA titers after oral administration (Fig. 4B). These data demonstrate that homology of the G proteins between vaccine strain and challenge virus does not necessarily yield the highest VNA titers and that the quantity of antibody largely depends on the ability to replicate sufficiently after a particular inoculation route.

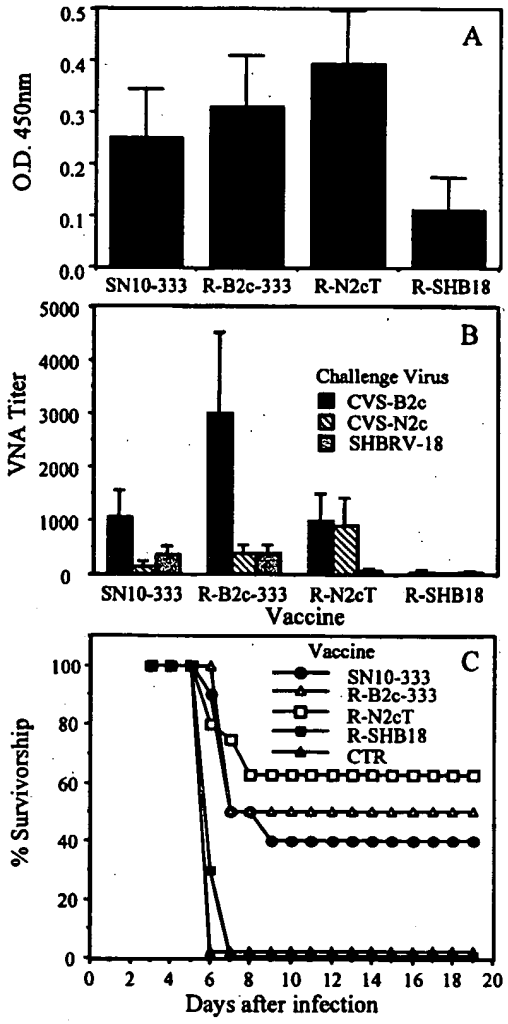

Fig. 4. Anti-RNP ELISA titers (A), VNA titers (B), and protection against i.c. challenge infection (C) in mice orally immunized with live rabies recombinant viruses. About 50 μl containing 10^6 FFU of SN10-333, R-B2c-333, R-N2cT, or R-5SHB18 was instilled into the buccal cavity of groups of ten mice, and mice were bled 2 weeks later. (A) AntiRNP ELISA titer was determined as described in Fig. 2A. (B) The mean titers of VNA against CVS-B2c, CVS-N2c, and SHBRV-18 were determined as described in Fig. 2B. (C) Groups of ten mice were immunized orally with 10^6 infectious recombinant virus particles or mock-immunized with saline (control) and 2 weeks later, infected i.c. with 100 LD_{50} of CVS-N2c. Mice were observed for 3 weeks and mortalities were recorded.

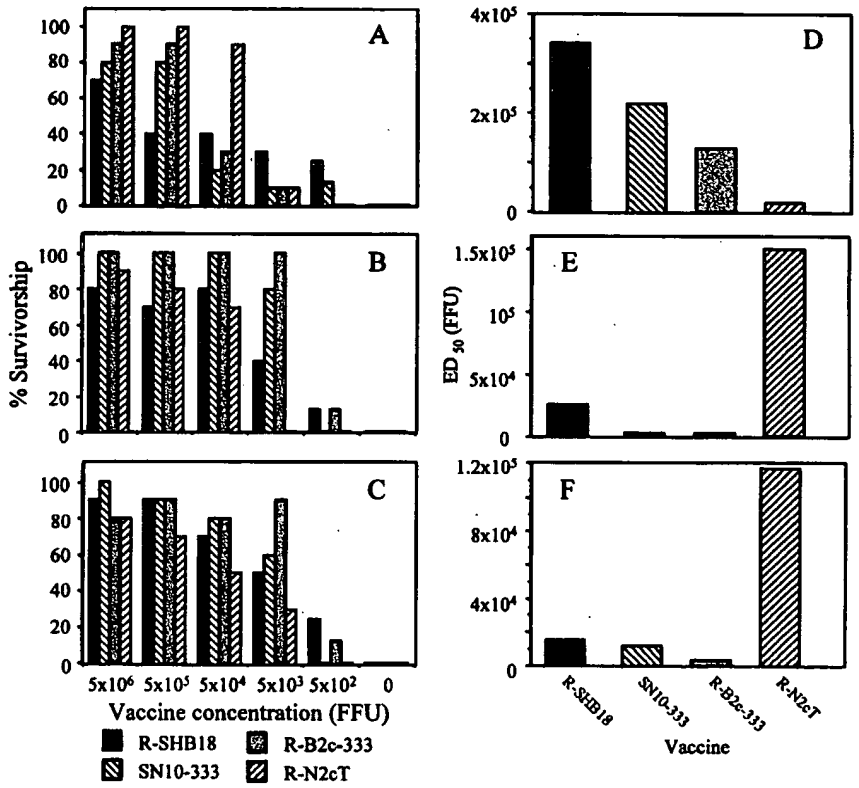

Fig. 5. (A–C) Survivorship in mice immunized with different recombinant vaccines and challenged intracranially with different rabies virus strains. Groups of ten 8-week-old Swiss Webster mice were immunized i.m. with different concentrations of R-SHB18, SNl0-333, R-B2c-333, and R-N2cT and 10 days after immunization, mice were infected i.c. with 100 LD_{50} of CVS-N2c (A); DRV-7 (B); or SHBRV-18 (C). Mice were observed for 4 weeks for clinical signs of rabies and mortalities were recorded. (D–F) The ED_{50} S was calculated for each vaccine from the mortality and survivorship rates in the different vaccination groups after i.c. challenge infection with the mouse-adapted CVS-N2c strain (D); the dog-associated DRV-7 strain (F); or the silver-haired bat-associated SHBRV-18 strain (F).

3.3. Protection conferred by infection with recombinant rabies viruses

To determine the level of protection against a lethal challenge conferred by the immune response to i.m. vaccination with SN10-333, R-B2c-333, R-N2cT, and R-SHB18, we used a modified NIH test for potency. After i.m. immunization, mice were challenged i.c. with three highly virulent rabies virus strains CVS-N2c, DRV-7, SHBRV18 (Fig. 5). Fig. 5A–C show the percent survivorship in groups of mice immunized i.m. with different doses of the recombinant viruses and infected i.c. with each of the three challenge viruses. In the case of a CVS-N2c challenge infection (Fig. 5A), the highest survivorship was found in the groups of mice immunized with homologous R-N2cT. The ED_{50} of R-N2cT immunization against CVS-N2c is 5, 10 and 16 times lower than the ED_{50} of R-B2c-333, SN-10-333, and R-SHB18, respectively (Fig. 5D), clearly demonstrating, in this instance, the superior protective activity of vaccination with homologous G with respect to the challenge virus. On the other hand, immunization with heterologous R-B2c-333, rather than homologous R-SHB18 resulted in the lowest mortality following challenge infection with SHBRV-18 (Fig. 5C). The ED_{50} of R-B2c-333 was 34, 3, and 6 times lower than those of R-N2cT, SN10-333, and R-SHB18, respectively (Fig. 5F). R-B2c-333 also conferred the best protection against challenge with the dog-associated rabies virus strain DRV-7 (Fig. 5B). Notably, as little as 5×10^3 virus particles of R-B2c-333 were sufficient to protect 100% of the animals against infection with DRV-7 while even 5×10^6 particles of R-5-SHB18 or R-N2cT conferred only incomplete protection. The results of these protection experiments clearly demonstrate marked differences in the potency of live virus vaccines, which are in some cases, but not always, related to G homology.

After oral immunization, the highest level of protection against i.c. challenge infection with CVS-N2c was seen in the group of mice that received the homologous R-N2cT vaccine (Fig. 4C). In contrast, none of the mice that were immunized with R-SHB18 survived the virus challenge. These vaccine protection data, which correlate with the VNA levels shown in Fig. 4B, demonstrate that highest levels of protection can be obtained if the antigenic makeup of the G protein of the vaccine virus is identical to that of the challenge virus.

4. Discussion

A number of effective killed and live attenuated rabies vaccines are currently available [4,26]. Nevertheless, large reservoirs of different rabies virus strains persist in wild and domestic animals [2,27]. The major reasons for this are difficulties, both technically and economically, in vaccinating these animals. For the most part, killed vaccines are not suitable for wild and stray animals because delivery of an appropriate antigenic mass is impossible to guarantee by the necessary baiting procedures. Only live vaccines can confer sufficient herd immunity to eliminate the reservoir. An ideal vaccine would protect against infection by all of the street rabies virus strains that are associated with different mammalian species in diverse geographical locations. As described here, antigenic differences between vaccine strains and the challenge viruses only become a critical factor in vaccine failure when low doses of vaccine are administered. Delivery of an appropriate vaccine dosage can only be guaranteed by parenteral administration. In this case, current vaccines licensed for use in humans and animals can all likely confer protective immunity against a variety of street rabies virus strains [28]. Since the amount of vaccine that is orally consumed cannot be easily controlled by bait delivery, protective immunity must be induced even when only a fraction of the vaccine dose is delivered. In order to achieve sufficient immunoprotection with only a minimal amount of vaccine, the following criteria must be fulfilled: (1) the vaccine virus should be able to replicate sufficiently in the recipient so that enough viral antigen is presented to the immune system; (2) the antigenic composition of the vaccine strain and the challenge virus should be closely related; and (3) the vaccine must confer sufficient protective immunity after oral immunization.

In addition to having optimal efficacy, safety is the most important criteria of any live vaccine. Since pathogenicity is not only a function of the virus but is also largely dependent on the site of infection and the immune status of the host, even the most attenuated rabies viruses can potentially cause a lethal encephalomyelitis [9,29]. As viral neuroinvasiveness is the major element of the pathogenesis of rabies, live rabies viruses suitable for vaccines must be crippled in this ability.

A major determinant of rabies virus pathogenicity involved in virus uptake is located at antigenic site III in G [10,12]. In an attempt to achieve further attenuation, site-directed mutagenesis was used to exchange the arginine at position 333 of G for glutamine. This substitution completely abolished the pathogenicity of SN10 and reduced that of R-B2c dramatically but had no effect on either the i.m. or i.c. pathogenicity of R-N2c [19]. This indicates that distinct pathogenic attributes are functional in different rabies viruses and targeting of a single determinant of pathogenicity is not sufficient to design a live vaccine strain. It has earlier been shown that a mismatch between the cytoplasmic domain of G and the RNP-M complex is sufficient to attenuate virus [19]. Replacement of the cytoplasmic domain of G from RN2c with that of SHBRV-18 (R-N2cT) rendered the virus apathogenic when administered via the intra-muscular route.

Rabies virus strains differ significantly in their ability to infect neuronal and nonneuronal cells [19,21]. This is likely to be reflected in quantitatively and qualitatively different immune responses to infection with various rabies virus strains. In this investigation we, therefore, utilized G from viruses with distinct neurotropisms to generate recombinant viruses, which retain the tissue specificity of the parent virus [19]. The fact that similar antibody responses to RNP were elicited by i.m. infection with these recombinants indicates that antigen production by these viruses is comparable. The overall quantities of neutralizing antibodies produced also appears to be equivalent, although there may be some differences in specificity for the in vitro challenge viruses. Importantly, the highest protection against a particular challenge virus was not necessarily dependent on G homology between vaccine strain and challenge virus. While vaccination with R-N2cT protected best against infection with CVS-N2c, R-B2c-333 and not R-SHB18, protected best against challenge with SHBRV-18. Neither the VNA response pattern nor homology between the Gs of the vaccine and challenge virus were incontrovertible indicators of protective immunity. For example, while i.m. vaccination with either R-SHB18 or R-N2cT induced comparable VNA levels against CVS-N2c, the protective effect of R-SHB18 was 16-fold less than R-N2cT. Conversely, i.m. vaccination with R-SHBV18 protected 4-fold less against challenge with SHBRV-18 than vaccination with R-B2c-333. These findings support the notion that there may be qualitative differences in protective capacity between different antibody populations as seen with monoclonal antibodies [30]. Alternatively, there may be differences in the induction of cell mediated immune mechanisms. In regard to oral immunization of free ranging animals, the results obtained from oral immunization experiments using recombinant vaccines in mice are promising. These data show that after i.m. immunization maximal protective immunity can be induced when the antigenic makeup of the G protein of the vaccine virus is identical to that of the challenge virus. On the other hand, these data also show that in order to be useful as an oral vaccine, the recombinant virus must be able to replicate sufficiently not only after parenteral inoculation but more importantly after oral administration.

The experience obtained with immunization of foxes in Europe with SAD-B19 indicates that natural reser-

voirs of rabies can be eliminated using live, non-neuroinvasive rabies viruses for vaccination. Conventional attenuated rabies vaccines, which have been derived from virulent strains by a selection procedure, consists of mixed populations of virus where minor constituents may retain pathogenicity. This has been demonstrated with CVS-24 which is composed of at least two variants with distinct pathogenic properties, CVS-B2c and CVS-N2c, each of which can be the dominant population depending upon the environment [20]. This may explain how conventional attenuated rabies vaccines may have occasionally caused unexpected lethal infections [5,31]. The possibility that a pathogenic variant will emerge in an engineered live recombinant vaccine is considerably more remote because the latter are derived from cloned cDNA with multiple, defined modifications. Using this approach, a recombinant rabies virus can be engineered to express the ectodomain of a G, which will induce a fully protective immune response in animals after peripheral challenge, yet not invade the CNS and cause lethal disease. This method will provide effective vaccines for particular rabies virus reservoirs. We also apply these results to our approach with RV-based vectors. We described a recombinant RV expressing HIV-1 gp160 in addition to the five other RV proteins [32]. Using the same approach we are currently constructing recombinant RV expressing G proteins from different RV strains and analyze, if this method will confer protective immunity against broader range of RV strains.

Acknowledgements

We thank Suchita Santosh Hodawadekar for excellent technical help. This work was supported by Public Health Service grants AI 45097 and AI 44340.

References

[1] Meslin FX, Fishbein DB, Matter HC. Rationale and prospects for rabies elimination in developing countries. In: Lyssaviruses, C.E. Rupprecht, B. Dietzschold, H. Koprowski (Eds.), Berlin: Springer, 1994;pp.1–26.

[2] Rupprecht CE, Smith JS, Fekadu M, Childs JE. The ascension of wildlife rabies: a cause for public health concern or intervention? Emerging Infect Dis 1995;1:107–14.

[3] Winkler WG, Bogel K. Control of rabies in wildlife. Sci Am 1992;266:86–92.

[4] Aubert MFA, Masson E, Artois M, Barrat J. In: Lyssaviruses CE, Rupprecht B, Dietzschold X, Koprowski H, editors. Oral Wildlife Rabies Vaccination Field Trials in Europe with Recent Emphasis on France. Berlin: Springer, 1994:19–243.

[5] Rupprecht CE, Charlton KM, Artois M, Casey GA, Webster WA, Campbell JB, Lawson KF, Schneider LG. Ineffectiveness and comparative pathogenicity of attenuated rabies virus vaccines for the striped skunk (*Mephitis mephistis*). J Wildlife Dis 1990;26:90–102.

[6] WHO Report of the 4th WHO consultation on oral immunization of dogs against rabies, Geneva. 1993. WHO/RabRes/93.42.

[7] Cox JH, Dietzschold B, Schneider LG. Rabies virus glycoprotein II. Biological and serological characterization. Infect Immun 1977;16:754–9.

[8] Dietzschold B, Ertl HC. New developments in the pre- and post-exposure treatment of rabies. Critical Rev Immunol 1991;10:427–39.

[9] Hooper DC, Morimoto K, Bette M, Weihe E, Koprowski H, Dietzschold B. Collaboration of antibody and inflammation in the clearance of rabies virus from the CNS. J Virol 1998;72:3711–9.

[10] Dietzschold B, Wunner WH, Wiktor TJ, Lafon M, Smith CL, Koprowski H. Characterization of an antigenic determinant of the glycoprotein which defines pathogenicity of fixed rabies virus strains. Proc Natl Acad Sci USA 1982;80:70–4.

[11] Morimoto K, Hooper DC, Spitsin S, Koprowski H, Dietzschold B. Pathogenicity of different rabies virus variants inversely correlates with apoptosis and rabies virus glycoprotein expression in infected primary neuron cultures. J Virol 1999;73:510–7.

[12] Seif I, Coulon P, Rollin PE, Flamand A. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J Virol 1985;53:926–34.

[13] Dietzschold B, Wiktor TJ, Trojanowski JQ, Macfarlan RI, Wunner WH, Torres-Anjel MJ, Koprowski H. Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus in vivo and in vitro. J Virol 1985;56:12–8.

[14] Lentz TL, Hawrot E, Wilson PT. Synthetic peptides corresponding to sequences of snake venom neurotoxins and rabies virus glycoprotein bind to the nicotinic acetylcholine receptor. Proteins Struct Funct Genet 1987;2:298–307.

[15] Thoulouze M-I, Lafage M, Schachner M, Hartmann U, Cremer H, Lafon M. The neural cell adhesion molecule is a receptor for rabies virus. J Virol 1998;72:7181–90.

[16] Tuffereau C, Benejean J, Blondel D, Kieffer B, Flammand A. Low-affinity nerve growth factor receptor (P7SNTR) can serve as a receptor for rabies virus. EMBO J 1998;17:7250–9.

[17] Mebatsion R, Konig M, Conzelmann K-K. Budding of rabies virus particles in the absence of the spike glycoprotein. Cell 1996;84:941–51.

[18] Mebatsion R, Weiland F, Conzelmann K-K. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J Virol 1999;73:242–50.

[19] Morimoto K, Foley HD, McGettigan JP, Schnell MJ, Dietzschold B. Reinvestigation of the role of the rabies virus glycoprotein in viral pathogenesis using a reverse genetics approach. J Neurol Virol 2000;6:373–81.

[20] Morimoto K, Hooper DC, Carbaugh H, Fu ZF, Koprowski H, Dietzschold B. Rabies virus quasispecies: Implications for pathogenesis. Proc Natl Sci USA 1998;95:3152–6.

[21] Dietzschold B, Morimoto K, Hooper DC, Smith JS, Rupprecht CE, Koprowski H. Genotypic and phenotypic diversity of rabies virus variants involved in human rabies: implications for postexposure prophylaxis. J Hum Virol 2000;3:50–7.

[22] Schnell MJ, Mebatsion T, Conzelmann K-K. Infectious rabies viruses from cloned cDNA. EMBO J 1994;13:4195–203.

[23] Wiktor TJ, MacFarlan RI, Foggin CM, Koprowski H. Antigenic analysis of rabies and Mokola virus from Zimbabwe using monoclonal antibodies. Dev Biol Stand 1984;57:199–221.

[24] Wilbur LA, Aubert MFA. In: Meslin F-X, Kaplan MM, Koprowski H, editors. Laboratory Techniques in Rabies. Geneva: World Health Organisation, 1996:360–8.

[25] Foley DF, McGettigan JP, Siler CA, Dietzschold B, Schnell MJ. A recombinant rabies virus expressing vesicular stomatitis virus

glycoprotein fails to protect against rabies virus infection. Proc Natl Acad Sci USA 2000;97:14680–5.
[26] Fu ZF, Dietzschold B, Plotkin SA, Rupprecht CE. In: Levine MM, Woodrow GC, Kaper JB, Cobon GS, editors. New Generation Vaccines, second edn. New York: Marcel Dekker, 1997:607–17.
[27] Smith JS, Orciari LA, Yager PA, Molecular epidemiology of rabies in the United States. Seminars in Virology 6 1995;387–400.
[28] Lodmell DL, Smith JS, Esposito JJ, Ewalt LC. Cross-protection of mice against a global spectrum of rabies virus variants. J Virol 1995;69:4957–62.
[29] Yang C, Jackson AC. Basis of neurovirulent rabies virus variant AvOl with sterotaxic brain inoculation in mice. J Gen Virol 1992;73:895–900.
[30] Dietzschold B. Antibody-mediated clearance of viruses from the mammalian central nervous system. Trends Microbiol 1993;1:63–6.
[31] Wandeler AI, Capt S, Kappeler A, Hauser R. Oral immunization of wildlife against rabies: concept and first field experiments, Rev Infect Dis 10 1988;Suppl. 4, 649–653.
[32] Schnell MJ, Foley HD, Siler CA, McGettigan JP, Dietzschold B., Pomerantz RJ. Recombinant rabies virus as potential live-viral vaccines for HIV-1, Proc Natl Acad Sci USA 97:3544–3549.